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NOX2 control over energy metabolism plays a role in acute myeloid leukaemia prognosis and survival

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ABSTRACT

Acute myeloid leukaemia (AML) is a highly heterogeneous disease, however the therapeutic approaches have hardly changed in the last decades. Metabolism rewiring and the enhanced production of reactive oxygen species (ROS) are hallmarks of cancer. A deeper understanding of these features could be instrumental for the development of specific AML-subtypes treatments. NADPH oxidases (NOX), the only cellular system specialised in ROS production, are also involved in leukemic metabolism control. NOX2 shows a variable expression in AML patients, so patients can be classified based on such difference. Here we have analysed whether NOX2 levels are important for AML metabolism control. The lack of NOX2 in AML cells slowdowns basal glycolysis and oxidative phosphorylation (OXPHOS), along with the accumulation of metabolites that feed such routes, and a sharp decrease of glutathione. In addition, we found changes in the expression of 725 genes. Among them, we have discovered a panel of 30 differentially expressed metabolic genes, whose relevance was validated in patients. This panel can segregate AML patients according to *CYBB* expression, and it can predict patient prognosis and survival. In summary, our data strongly support the relevance of NOX2 for AML metabolism, and highlights the potential of our discoveries in AML prognosis.

1. Introduction

Acute myeloid leukaemia (AML) is the most frequent acute leukaemia in adults with an incidence of 3–5 cases per 100,000 population per year [1]. AML is highly heterogeneous with more than 250 different mutations and 20 cytogenetic alterations identified [2]. Despite that, the therapeutic approach has not changed substantially since the 1970s, being the so-called "7 + 3" regimen of anthracycline and cytarabine, the most common approach [3]. A deeper knowledge of the disease will be instrumental towards the development of more specific AML-subtype treatments.

Many cancer cells show a distinctive metabolism, characterised by a preference for the glycolysis over oxidative phosphorylation (OXPHOS) [4]. However, recent data reveal that tumour cells are extraordinarily plastic metabolically, so cancer metabolism goes far beyond the Warburg effect [5]. Besides, tumour cells show an elevated level of reactive oxygen species (ROS), which can contribute to tumour transformation [5,6].

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Originally regarded as toxic by-products, ROS are now acknowledged as key signalling regulators [6,7]. Haematopoietic stem cells (HSCs) show a low level of ROS, which seems indispensable for keeping their quiescence [8], while an increase in ROS level is required for haematopoietic differentiation [9]. A glycolytic metabolism in HSCs would contribute to maintain ROS levels low, while OXPHOS activation would induce the level of ROS to rise during differentiation. Unlike HSCs, AML leukemic stem cells (LSKs) are OXPHOS dependent [10], a feature also shared by chemoresistant cells [11]. In contrast, AML blasts in most cases display the canonical Warburg effect despite having unrestricted access to oxygen [12]. However, the scenario is not that simple, there are some AML blasts that are OXPHOS dependent [13]. This just give us a glimpse about the complexity of metabolism in leukaemia and uncovers the necessity for further studies.

Metabolism alteration and enhanced ROS production are acknowledged as hallmarks of cancer that are intimately intermingled [5]. Metabolism is one of the main sources of ROS, and at the same time it can be under redox control, for instance through the regulation of certain metabolic enzymes by reversible oxidation [5].

Redox signalling depends on an adjustable source of ROS such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX). Unlike all the other cellular systems that produce ROS as secondary by-products, NOXs are specialised in the production of ROS, and their activity can be regulated [14]. The founding member of the family is the phagocyte oxidase or NOX2, required for the immune response [15].

Increasing evidence relate NADPH oxidases with the regulation of metabolism, especially NOX2 [16–18], and NOX4 [19–21]. Moreover, in AML, NOX2 has been associated with enhanced production of ROS and metabolism regulation [22], and with the transference of mitochondria from stromal cells to leukemic blasts [23]. We have recently shown that *CYBB* (NOX2 coding gene) shows a variable expression in AML patients, so patients can be classified based on such difference. In addition, *CYBB* is correlated with the expression of 28 metabolism involved genes, which suggest the implication of NOX2 in the regulation of metabolism. In addition, this panel of genes can be used for patient classification and survival prediction [24]. Along the same line, other authors have also suggested the relevance NOX2 as a prognosis factor in AML [25].

Bearing in mind the variable *CYBB* expression in AML patients, here we have analysed whether NOX2 levels are important for AML metabolism control. We show that the lack of NOX2 in THP-1 cells slowdowns glycolysis and OXPHOS. NOX2 deletion induces the accumulation of glycolysis and tricarboxylic acid (TCA) cycle precursors, and unexpectedly a sharp decrease of glutathione. Metabolism alterations upon NOX2 deletion are also reflected at the transcriptome level, which allowed us to discover a group of 30 metabolic genes, which relevance was validated by patient sample analysis. These genes can segregate AML patients according to *CYBB* expression, and more importantly, they can predict patient prognosis group and survival. In summary, our data strongly support the relevance of NOX2 for AML metabolism, and highlights the potential of NOX2 and metabolism in AML prognosis.

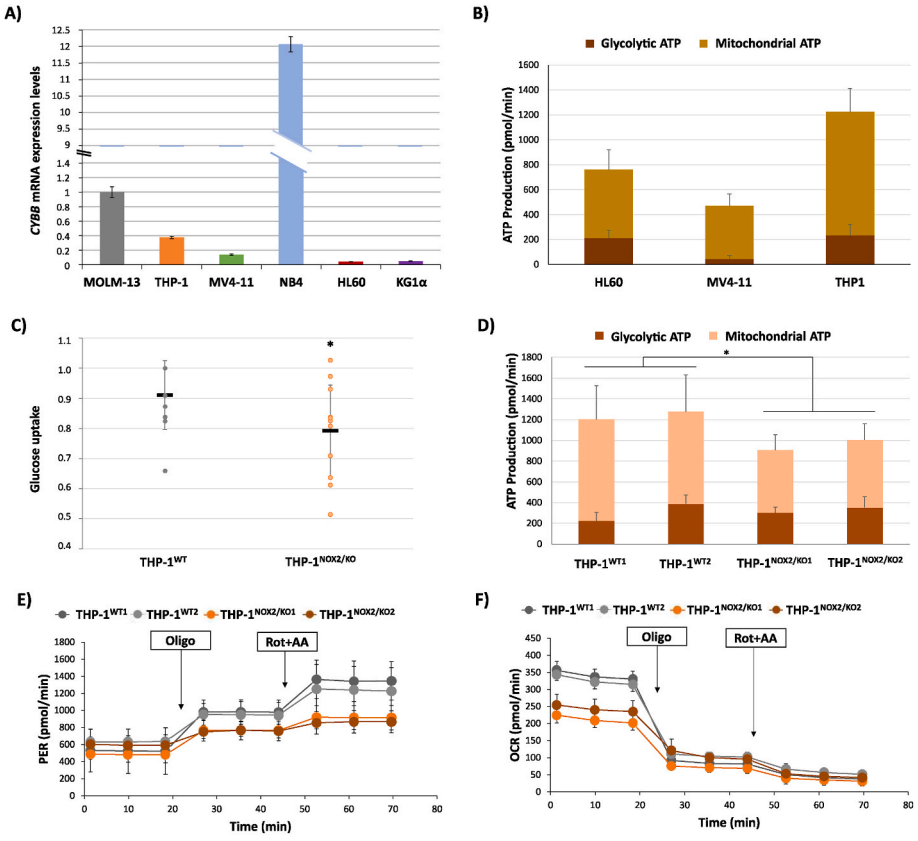

Fig. 1. NOX2 expression levels affect energy metabolism of AML cell lines. A) Levels of *CYBB*, the gene encoding NOX2, were measured by RT-qPCR (n = 3). The cell lines appeared in decreasing order of FAB classification, i.e. from highest to lowest maturation status. B) Ratio of glycolytic and mitochondrial ATP production in HL60 (n = 8), MV4-11 (n = 5) and THP-1 (n = 5) cells. C) Glucose uptake measured by 2-NBDG label of wild type (THP-1^{WT}) and NOX2 deletion THP-1 cell clones (THP-1$^{NOX2/KO}$) (n = 5). D) Levels of glycolytic and mitochondrial ATP production in THP-1^{WT} and THP-1$^{NOX2/KO}$ (n = 5). E) Proton efflux rate (PER) of THP-1^{WT} and THP-1$^{NOX2/KO}$. F) Oxygen consumption rate (OCR) of THP-1^{WT} and THP-1$^{NOX2/KO}$. The data in E and F show the results of one representative experiment out of 5 in total. Results are shown as mean ± standard deviation and are considered statistically significant changes with respect to control cells when *p < 0.05. Oligo: oligomycin; Rot: rotenone; AA: actinomycin A.

2. Results

2.1. NOX2 expression level affects energy metabolism in AML cells

The expression of *CYBB* was analysed in several AML model cell lines. As reported for patients [24], *CYBB* expression is highly variable (Fig. 1A). NB4 cells, an acute promyelocytic leukaemia (APL) cell line, showed the highest *CYBB* expression, while in the other cell lines *CYBB* increased along with the degree of differentiation (Fig. 1A), as previously suggested [24,26].

To test whether *CYBB* expression determines metabolism we analysed three cell lines with differential *CYBB* expression, from lower to higher: HL60, MV4-11 and THP-1. No obvious correlation was observed between *CYBB* expression and total ATP production. THP-1 and MV4-11 cells showed the highest and lowest ATP production respectively and HL60 cells, which had the lowest *CYBB* expression, obtained more ATP from glycolysis than THP-1 cells (Fig. 1B). We selected THP-1 for further experiments as they present the most active metabolism.

Silencing NOX2 in THP-1 cells did not seem to alter mitochondrial ATP production (Supplementary Fig. 1A), but it led to an increase in membrane potential (Supplementary Fig. 1C) and mitochondrial ROS (Supplementary Fig. 1D), together with an increase in complex III and V protein levels (Supplementary Fig. 1E). This phenotype suggests a forced mitochondrial metabolism to maintain ATP production, suggesting that NOX2 might affect mitochondrial function.

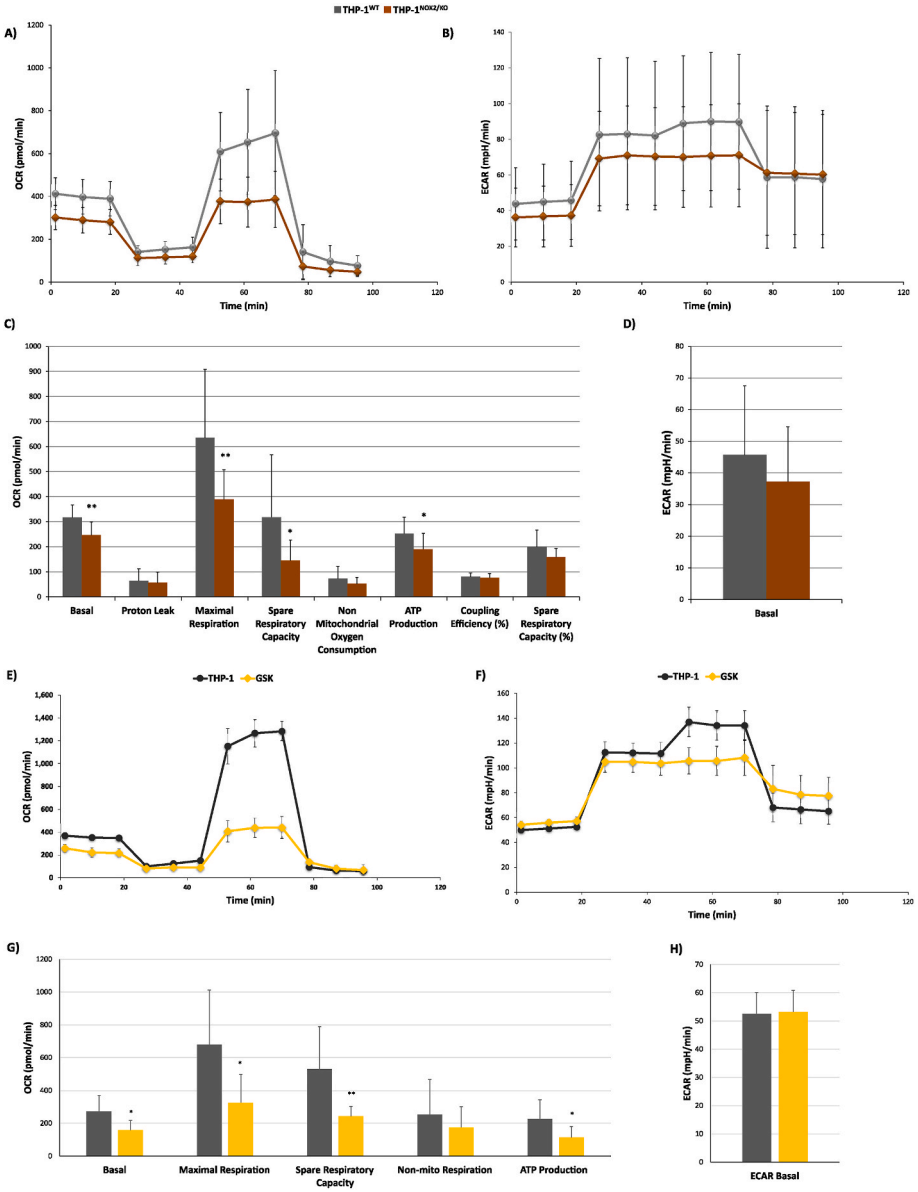

Fig. 2. NOX2 deletion or inhibition in THP-1 cells results in a lower basal energy metabolism. Mito Stress Assay (Agilent) was performed for NOX2-deleted THP-1 cells (THP-1$^{NOX2/KO}$) and their wild type counterparts (THP-1^{WT}): A) Oxygen Consumption Rate (OCR) profile of THP-1$^{NOX2/KO}$ and THP-1^{WT} cells. B) Extracellular Acidification Rate (ECAR) profile of THP-1$^{NOX2/KO}$ and THP-1^{WT} cells. C) Quantification of mitochondrial metabolism parameters of THP-1$^{NOX2/KO}$ and THP-1^{WT} cells (n = 13). D) Basal Extracellular Acidification Rate of THP-1$^{NOX2/KO}$ and THP-1^{WT} cells. Mito Stress Assay (Agilent) was also performed with THP-1 cells treated with or without 100 μM GSK2795039 for 16h: E) Oxygen Consumption Rate (OCR) profile. F) Extracellular Acidification Rate (ECAR) profile. G) Quantification of mitochondrial metabolism parameters (n = 6). H) Basal Extracellular Acidification Rate (n = 6). Results are shown as mean ± standard deviation and are considered statistically significant changes with respect to control cells when *p < 0.05, **p < 0.01 and ***p < 0.001. Oligo: oligomycin; Rot: rotenone; AA: actinomycin A.

2.2. NOX2 deletion slowdowns energy metabolism and alters fuel usage

CYBB expression was knocked out in THP-1 cells (THP-1$^{NOX2/KO}$) (Supplementary Fig. 2). THP-1$^{NOX2/KO}$ cells showed a significant decrease in glucose uptake (Fig. 1C) and ATP production (Fig. 1D). THP-1$^{NOX2/KO}$ cells showed a lower proton efflux rate (PER) (Fig. 1E) and oxygen consumption rate (OCR) profiles (Fig. 1F), suggesting that both, glycolysis and OXPHOS are affected by NOX2 removing.

Further analyses showed that THP-1$^{NOX2/KO}$ displayed a reduced mitochondrial metabolism (Fig. 2A–C). Very similar results were observed upon chemical inhibition of NOX2 with GSK2795039 (Fig. 2E–G). Both experimental approaches led to a reduced mitochondrial respiration, with significant decreases in basal respiration, maximal and spare respiratory capacity, and in ATP production.

We next tested whether the use of fuels changed upon NOX2 deletion. Control cells were more sensitive than THP-1$^{NOX2/KO}$ cells to inhibition of pyruvate transport to the mitochondria with UK5099, so THP-1$^{NOX2/KO}$ cells displayed a higher maximal respiration ratio (Fig. 3A and B). Upon inhibition of glutaminase with BPTES, THP-1$^{NOX2/KO}$ cells tended to show a lower maximal respiration ratio than control, although not reaching statistical significance (Supplementary Figs. 3A–B). The effect of etomoxir (an inhibitor of carnitine palmitoyl-transferase 1a -CPT1a-) was also tested; THP-1$^{NOX2/KO}$ cells tended to show a more pronounced acute response to this inhibitor than control cells, although not reaching statistical significance (Supplementary Figs. 3C–D). This would suggest that THP-1$^{NOX2/KO}$ cells would be more dependent on fatty acid oxidation than control cells, and it could be therefore surmised that NOX2 affects fuel usage. To test this hypothesis further, the use of palmitate was analysed after an overnight starvation to force lipid metabolism. The acute response to etomoxir treatment was significantly more pronounced in THP-1$^{NOX2/KO}$ cells (Fig. 3C–E), which is in agreement with the results presented in Supplementary Fig. 3D. These results support the notion that NOX2 deletion enables cells to activate lipid metabolism more efficiently, although such metabolism does not seem to compensate the reduced use of glucose in basal conditions, since NOX2 deletion leads to a decrease in ATP production (Fig. 1D). In contrast, control cells rely more on glucose metabolism, since they were more severely affected by UK5099 treatment (Fig. 3A and B).

2.3. NOX2 deletion alters the metabolome of AML cells

We next analysed whether NOX2 deletion induces changes at the

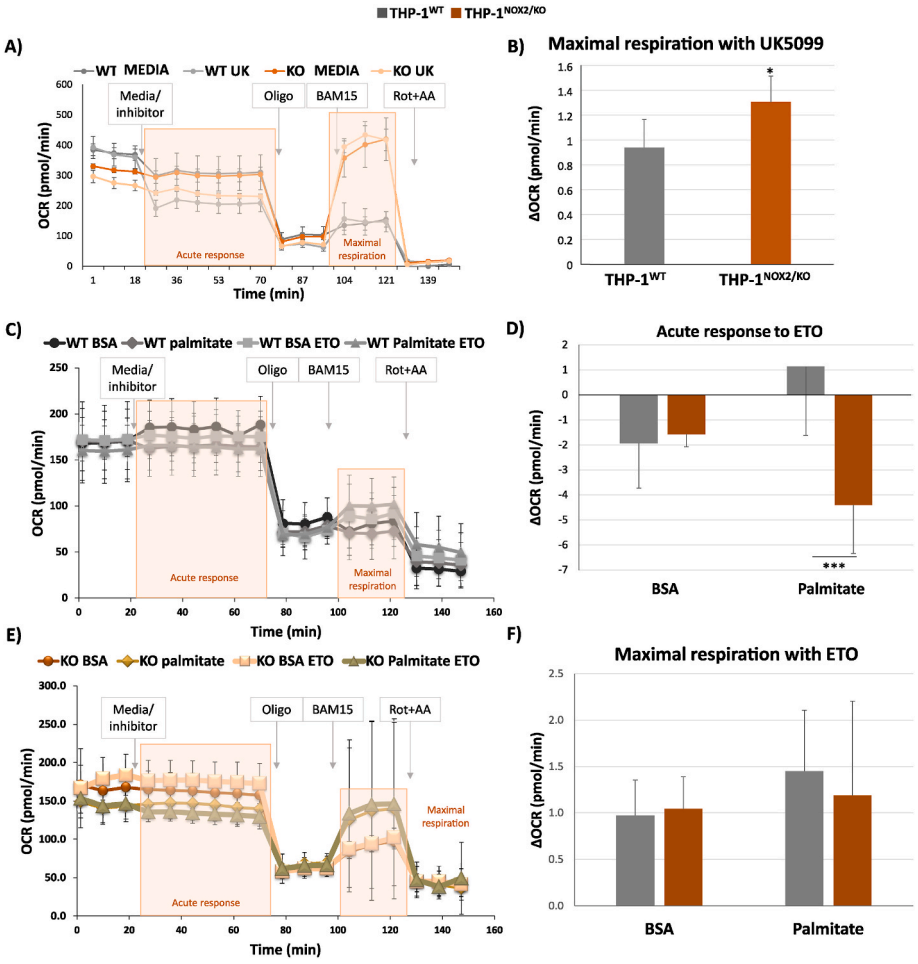

Fig. 3. NOX2 deletion in THP-1 cells enables a more efficient fatty acids metabolism on a par with a lower use of glucose/pyruvate energy source. Using the XF Substrate Oxidation Stress Test kit (Agilent), mitochondrial respiration of NOX2-deleted THP-1 cells (THP-1$^{NOX2/KO}$) and their wild type counterparts (THP-1^{WT}) after treatment with UK5099 or Etomoxir was determined. A) Oxygen Consumption Rate (OCR) after UK5099 (n = 7). B) Maximal respiration ratio calculated as the proportion of either THP-1^{WT} nor THP-1$^{NOX2/KO}$ treated with UK5099 compared to their untreated counterpart after BAM15 injection (n = 7). C) Oxygen Consumption Rate (OCR) of THP-1^{WT} after Etomoxir with and without palmitate supply (n = 10). D) Acute respiration ratio calculated as the proportion of either THP-1^{WT} nor THP-1$^{NOX2/KO}$ treated with Etomoxir compared to their untreated counterpart (n = 10). E) Oxygen Consumption Rate (OCR) of THP-1$^{NOX2/KO}$ after Etomoxir with and without palmitate supply (n = 10). F) Maximal respiration ratio calculated as the proportion of either THP-1^{WT} nor THP-1$^{NOX2/KO}$ treated with Etomoxir compared to their untreated counterpart after BAM15 injection (n = 10). Results are shown as mean ± standard deviation and are considered statistically significant changes with respect to control cells when *p < 0.05. Oligo: oligomycin; Rot: rotenone; AA: actinomycin A; ETO: etomoxir; UK: UK5099.

metabolome level (Supplementary Tables 1 and 2). Principal component analysis (PCA) revealed a homogeneous behaviour of the samples within groups (Supplementary Fig. 4A). Orthogonal partial least square discriminant analysis (OPLSDA) was performed to separate samples according to their metabolites content, which revealed a homogeneous metabolite content within both groups, and distinctive between them (Fig. 4A and Supplementary Fig. 4B).

THP-1$^{NOX2/KO}$ cells showed a higher concentration of metabolites that can feed both glycolysis (glucose) and TCA cycle (lactate, acetate, asparte, glutamine and glutamate) (Fig. 4B, Supplementary Figs. 4C–E and Supplementary Table 2). The accumulation of such metabolites is consistent with a slowing of these catabolic pathways upon NOX2 deletion as suggested above by the Seahorse analyses. In the same line, THP-1$^{NOX2/KO}$ cells showed an accumulation of two aspartate derivatives (*N*-methyl-D-aspartate (NMDA) and *N*-Acetylaspartate (NAA)). Of note, NAA has been proposed as a reservoir of acetate for acetyl coenzyme A synthesis [27]. Its accumulation would also agree with the TCA cycle slowdown. Moreover, a reduced use of glucose could explain the decrease in myoinositol observed in THP-1$^{NOX2/KO}$ cells, a metabolite that is synthesised from glucose-6-phosphate.

Surprisingly THP-1$^{NOX2/KO}$ cells showed a higher concentration of ATP and phosphocreatine, consistent with a decreased ADP level (Supplementary Fig. 4C and Supplementary Table 2), which seemed controversial with catabolism slowdown, fuel accumulation and with the lower ATP production rate displayed by these cells in the seahorse analyses (Fig. 1D). It has been previously reported that upon restriction on the energy producing routes, cells are capable of activating mechanism for reducing ATP consumption [28]. Therefore, we wonder whether this mechanism is activated in THP-1$^{NOX2/KO}$ cells.

2.4. NOX2 deletion leads to glutathione depletion and stabilisation of NRF2

The metabolomics analyses also evidenced a reduction of glutathione (GSH) in THP-1$^{NOX2/KO}$ cells (Supplementary Table 2). We analysed this issue further and found that GSH levels in THP-1$^{NOX2/KO}$ cells were virtually depleted (Fig. 5A–C). Inhibition of NOX2 with two different chemical inhibitors (GSK2795039 and APX-115) also led to a significant decrease in GSH and GSSG cellular levels (Supplementary Fig. 5). The ability of the cell to metabolise GSH and GSSG is not compromised according to the analysis of glutathione reductase (GR) (Fig. 5D) and glutathione peroxidase (GPX) (Fig. 5E). In fact, GR activity was enhanced, perhaps to compensate the dramatic loss of GSH. Such loss could be due to the inhibition of the route of synthesis, or an increased GSH efflux and/or degradation [29]. The first alternative would agree with the accumulation of GSH parent compounds, glycine and glutamate, observed in THP-1$^{NOX2/KO}$ cells. Cysteine content was not altered in THP-1$^{NOX2/KO}$ cells, however we observed an increase in taurine, a non-essential amino acid that can be synthesised from cysteine. Therefore, it is likely that THP-1$^{NOX2/KO}$ cells divert cysteine towards taurine synthesis to the detriment of glutathione synthesis.

In addition to GSH depletion, superoxide dismutase activity (SOD) was decreased in THP-1$^{NOX2/KO}$ cells (Fig. 5F), probably due to the decrease in SOD1 (Cu/Zn SOD), because no changes in SOD2 (MnSOD) were observed (Fig. 5G).

An early response to oxidative stress is the stabilisation of nuclear factor-erythroid 2 p45-related factor 2 (NRF2), a transcription factor that activates the expression of antioxidants and detoxifying enzymes [30]. We observed that THP-1$^{NOX2/KO}$ cells showed a significant increase in NRF2 levels (Fig. 5H).

2.5. CYBB deletion enhances mTOR activation and β-CATENIN stabilisation

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signalling pathway is deeply involved in metabolism control [21]. Although p-AKT/AKT seemed invariable (Fig. 6A), THP-1$^{NOX2/KO}$ cells showed a strong increase in mTOR levels accompanied by an increase in its phosphorylated form (Fig. 6B). mTOR pathway upregulation was confirmed by the enhanced activation of ribosomal protein S6 kinase (p70S6K) (Fig. 6C).

Glycogen synthase kinase-3 beta (GSK3β)/β-CATENIN have been involved in the regulation of glucose metabolism, protein synthesis, mitochondrial biogenesis and metabolism, glutamine and lipid metabolism and glycogen anabolism [31]. GSK3β is subjected to regulation by phosphorylation at different residues and by several kinases. Ser-9 phosphorylation leads to GSK3β inhibition [32]. This residue is a p70S6K target [33], and in agreement with p70S6K activation THP-1$^{NOX2/KO}$ cells showed a higher level of GSK3β/Ser-9 phosphorylation (Fig. 6D). Accordingly, β-CATENIN levels were enhanced upon NOX2 deletion (Fig. 6D).

The entry of glucose catabolism in the TCA cycle is mediated by pyruvate dehydrogenase complex (PDC). PDC is subjected to regulation by allostery and phosphorylation, the later leading to inhibition when pyruvate dehydrogenase E1α subunit (PDHE1α) is phosphorylated at several serine residues by pyruvate dehydrogenase kinase (PDK) [34]. PDK expression can be activated by β-CATENIN accumulation [35], and in agreement with β-CATENIN stabilisation, we observed an increase in PDK2 levels (Fig. 6E). PDHE1α/Ser-293 phosphorylation was enhanced in THP-1$^{NOX2/KO}$ cells (Fig. 6F), consistent with a decreased PDC activity, and with the TCA cycle slowdown observed in these cells (Figs. 1F and 2).

In summary, intracellular signalling changes upon NOX2 deletion might be determinant for the metabolism rewiring observed in THP-1 cells.

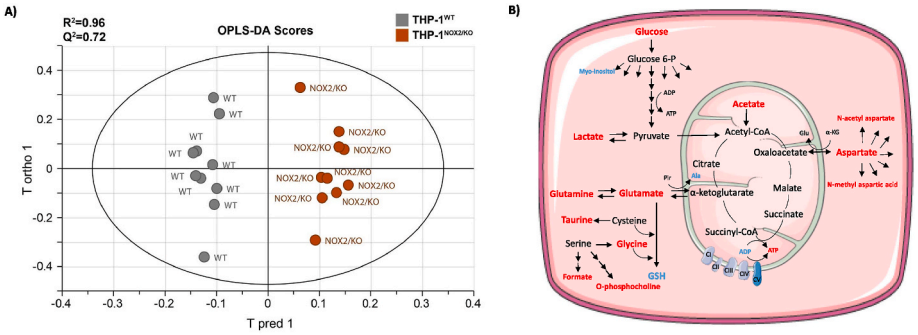

Fig. 4. NOX2 deletion in THP-1 cells alters the metabolome. The metabolome of NOX2-deleted THP-1 cells (THP-1$^{NOX2/KO}$; n = 10) and their wild type counterparts (THP-1^{WT}; n = 10) was studied by NMR. A) OPLS-DA score plot from analysis of the full 1H NMR profiles (1 predictive +3 orthogonal components, CV-ANOVA $p = 0.013$). B) Graphical abstract of the altered metabolites. In blue the metabolites that showed lower levels and in red the metabolites with increased levels in the THP-1$^{NOX2/KO}$ versus THP-1^{WT} comparison.

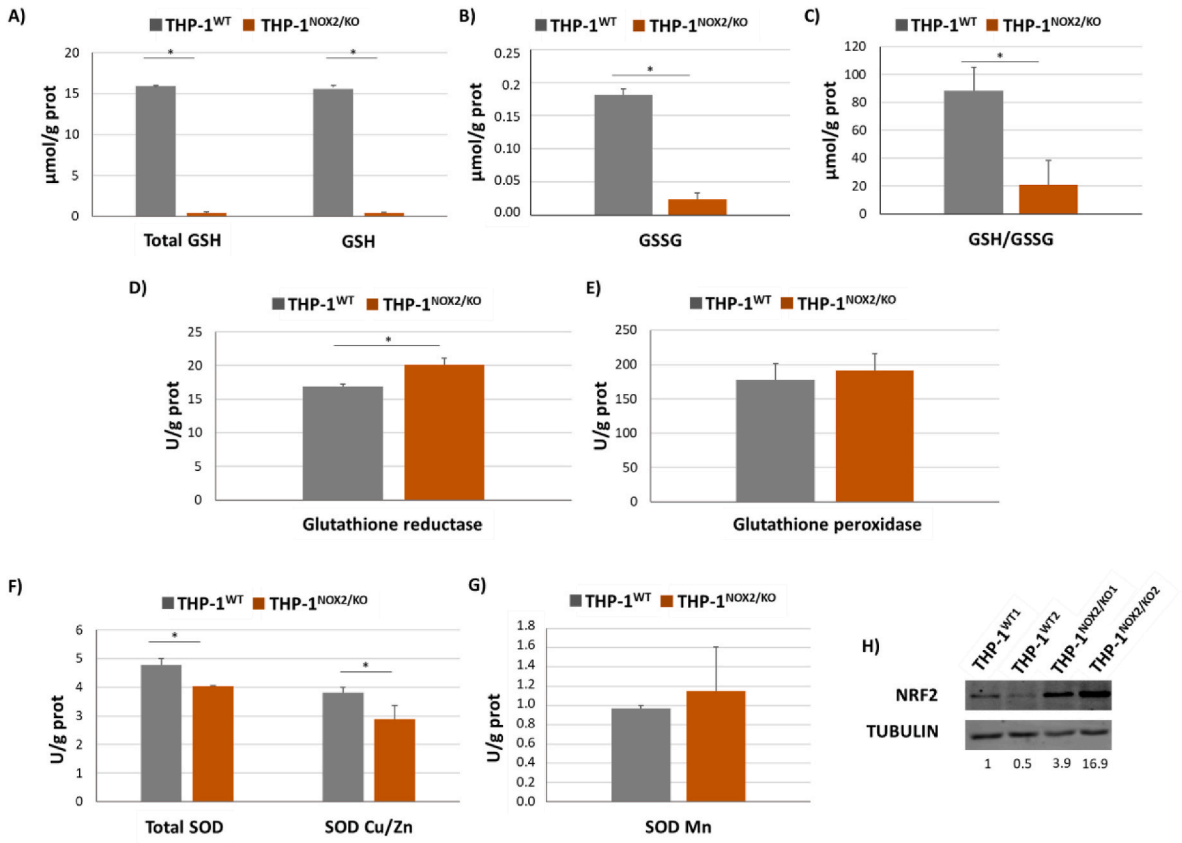

Fig. 5. NOX2 deletion compromises antioxidant system in THP-1 cells. Glutathione and superoxide dismutase antioxidant systems and NRF2 levels were measured in NOX2-deleted THP-1 cells (THP-1$^{NOX2/KO}$; n = 3) and their wild type counterparts (THP-1^{WT}; n = 3). A) Total GSH levels representing the sum of GSH and GSSG and GSH alone. B) GSSG levels. C) GSH/GSSH ratio. D) Enzymatic activity of glutathione reductase. E) Enzymatic activity of glutathione peroxidase. F) Total superoxide dismutase (SOD) enzyme activity representing the sum of SOD1 (bound to Cu/Zn) plus SOD2 (bound to Mn) and SOD1 alone. G) Enzymatic activity of SOD2. H) NRF2 protein levels by Western Blot (n = 4).

2.6. Transcriptome analysis suggests the alteration of lipid metabolism upon NOX2 deletion

To gain further insight into the consequences of NOX2 deletion, we performed transcriptome analyses in THP-1$^{NOX2/KO}$ and control cells (SRA database, PRJNA977859). We identified 725 differentially expressed genes (DEGs) in THP-1$^{NOX2/KO}$ cells, 490 upregulated and 235 downregulated (Supplementary Excel File, Fig. 7A and B and Supplementary Table 3). Gene ontology (GO) analysis (Fig. 7C) showed enrichment in biological processes (BP) related with the immune response and angiogenesis. Among cellular components (CC), terms related with extracellular matrix and with the vesicle secretory pathway were mostly enriched. Finally, among molecular functions (MF) there were several terms regarding transcription activators and RNA polymerase II function, myosin binding and tyrosine kinase receptor function. We also looked at enriched pathways by the KEGG database. Attending to the gene ratio, the pathway more severely affected by NOX2 deletion was *lipid and atherosclerosis* (Fig. 7D). Next, we found *Cytokine-Cytokine receptor interaction*, which may be related with the importance of NOX2 for signalling processes. And next, *Transcriptional misregulation in cancer*, suggesting the alteration of transcription process (Fig. 7C). If we analyse globally the 20 KEGG pathways shown in Fig. 7D, 7 are related with cell signalling, 7 with diseases and inflammation, 3 with lipid metabolism, 2 with cell differentiation, and 1 with transcription. Considering the higher dependence of THP-1$^{NOX2/KO}$ cells on palmitate oxidation (Fig. 3), it is remarkable that three KEEG pathways are related with lipid metabolism (*lipid and atherosclerosis, regulation of lipolysis in adipocytes, alcoholic liver disease*). Along the same line, a careful analysis of the transcriptomic data revealed a list of 12 genes involved in lipid catabolism, which are overexpressed in THP-1$^{NOX2/KO}$ cells (Supplementary Table 4). In summary, this transcriptomic analysis strongly suggests that NOX2 deletion induce lipid metabolism rewiring in AML cells.

2.7. Transcriptomic differences observed upon NOX2 deletion in THP-1 cell line can be harnessed for AML patient classification and survival prediction

We have recently described a gene signature of 28 metabolic genes correlated to *CYBB* (29G) with extraordinary prognostic value in AML [24]. Considering this, it is reasonable to surmise that NOX2 can modulate metabolism through the regulation of gene expression, including the 28 metabolic genes we described before [24], and other genes. The identification of such genes would provide further insights regarding AML metabolism control.

To address this issue we tested whether the transcriptome alterations found in THP-1$^{NOX2/KO}$ cells are meaningful at the patient setting, and whether such differences have any prognosis value. Using a list of 941 metabolism involved genes, we looked for metabolic genes within the DEGs detected in the comparison between THP-1$^{NOX2/KO}$ and THP-1^{WT} cells, and found a list of 30 genes (Supplementary Table 5 and Supplementary Fig. 6), interestingly 3 of them belonging to 29G (Supplementary Table 5). Compellingly, these 30 genes could segregate AML patients according to *CYBB* expression (Fig. 8A). This further supports the tight connection *CYBB*-metabolism, not only in THP-1 cell line, but also in AML patient samples. Such result was validated in two additional datasets (Supplementary Fig. 7). Next, we tested whether these 30 DEGs have any prognosis value, finding that they could segregate with great

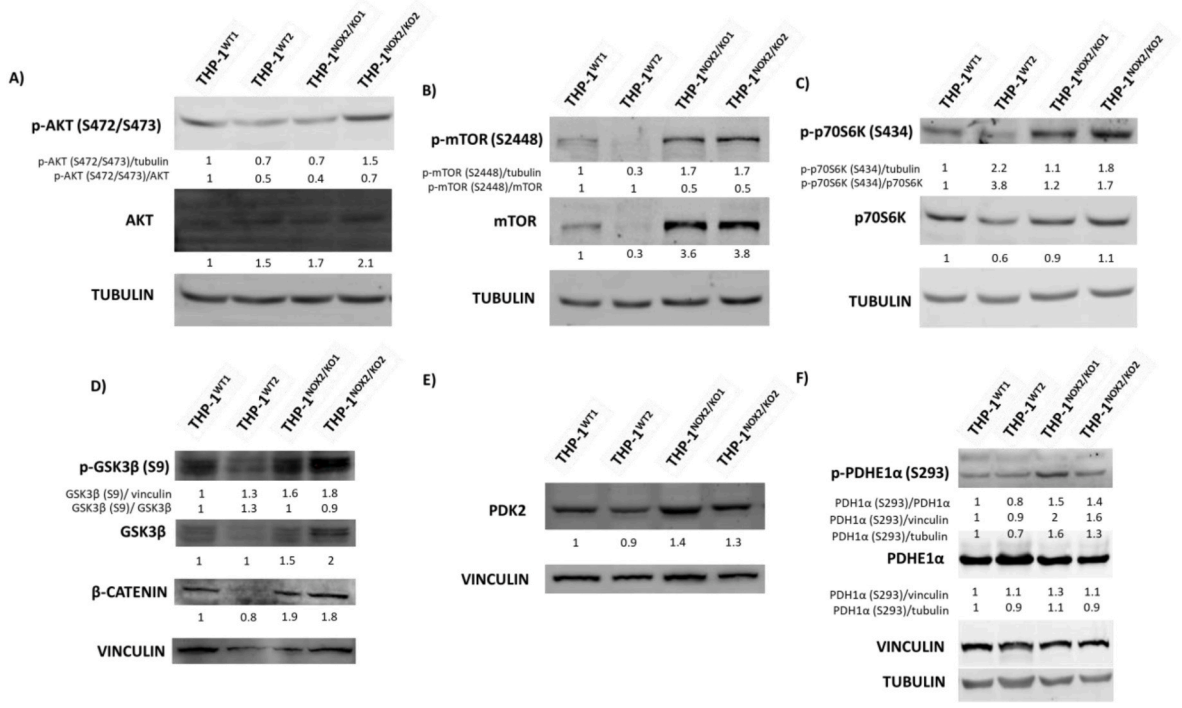

Fig. 6. NOX2 deletion activates mTOR pathway, stabilises β-CATENIN and leads to PDH inactivation in THP-1 cells. The main proteins involved in the mTOR and β-CATENIN pathways and PDK complex were quantified by Western blot in NOX2-deleted THP-1 (THP-$1^{NOX2/KO}$) and wild type (THP-1^{WT}) cells. A) AKT and its activating phosphorylated form at Ser472 or Ser473. B) mTOR and its activating phosphorylated form at Ser2448. C) p70S6K and its activating phosphorylated form at Ser434. D) GSK3β and its inactivating phosphorylated form at Ser9 and β-CATENIN. E) PDK2. F) PDHE1α and its inactivating phosphorylated form at Ser293. Representative images from at least 3 independent experiments are shown.

feasibility the three AML prognosis groups, and the healthy controls (Fig. 8B and C), which was validated in an additional dataset (Supplementary Fig. 8A). Finally, we computed an Expression Index (EI) [24], and tested whether it could predict patient overall survival (OS). Our results show that patients with low EI have a significantly higher OS (Fig. 8D), which was validated in a different dataset (Supplementary Fig. 8B). 30G displays similar global accuracies to our previously described 29G gene signature [24] (Supplementary Table 6). Whereas 30G classifies better good prognosis patients, 29G does so with poor prognosis patients.

In summary, these results strongly support the relevance of *CYBB* expression for AML metabolism control, and evidence metabolism as a powerful prognosis tool.

3. Discussion

Oxidative stress is intimately linked to metabolism rewiring in cancer, and compelling evidence support the relevance of NADPH oxidases, the only specialised cellular source of ROS, in metabolism control[16, 36]. Our previous results show that AML patients can be stratified according to *CYBB* expression, which at the same time correlates with the expression of 28 metabolic genes [24]. This strongly suggested that AML metabolism may depend on NOX2 levels, and here we have tested this hypothesis and dissect its contribution.

Depletion of NOX2 expression in THP-1 cells significantly reduced ATP production, and both, glycolysis and OXPHOS were downregulated. This notion was further supported by metabolome analysis, which in line with the slowdown of glycolysis and OXPHOS showed an accumulation of substrates that feed these catabolic pathways. NOX2 activity has been previously associated with the activation of glycolysis in neutrophils [18], AML blast cells [22], and AML cell lines [17]. Robinson et *al.* have previously suggested that ROS produced by NOX2 are responsible for the induction of glycolysis [22]. In agreement with that, NOX2 deletion in THP-1 cells led to a decreased glycolytic activity. However, our results points to OXPHOS as the main source of energy in terms of amount of ATP produced. OXPHOS metabolism is significantly reduced upon NOX2 deletion or inhibition, which suggests that NOX2 activity would be required to maintain mitochondrial activity in AML. We have recently found that silencing of NOX2 in chronic myeloid leukaemia (CML) cells induces *NOX4* expression, which eventually leads to an increased mitochondrial activity and stress [21]. Therefore, it can be surmised that the role of NOX2 in metabolism control will depend on the cellular context. In AML, NOX2 activity would be required to sustain an active energy metabolism at the glycolytic and OXPHOS level, while in CML NOX2 by regulating *NOX4* expression, contributes to maintain the Warburg effect activated by BCR-ABL.

Our results are consistent with the notion that NOX2 deletion reprograms THP-1 cells metabolism, enabling lipid metabolism activation more efficiently. While control cells were more affected by glucose metabolism inhibitor, cells without NOX2, were more affected by fatty acid metabolism inhibitor as happens for LSC [37]. This would imply that NOX2 would promote the use of glucose over fatty acids. A lower use of the two latter fuels would allow AML cells to keep a high TCA cycle activity and anabolic processes such amino acids and lipid synthesis. If this hypothesis were true, NOX2 deletion would also hamper anabolism. In line with such hypothesis THP-$1^{NOX2/KO}$ cells showed the accumulation of phosphocholine, a key intermediate in the biosynthesis of membrane lipids (phosphatidylcholines and sphingomyelins) [38]; formate, required by folate coenzymes as one-carbon donor for nucleic acid synthesis or mitochondrial proteins synthesis [39]; and N-acetyl aspartate, which has been involved in lipid synthesis [32].

A surprising result was the reduction of glutathione levels upon NOX2 deletion or inhibition. A very similar result has recently been reported for NOX1, which deletion in cardiomyocytes induces glutathione depletion; the authors suggest that it might be due to the upregulation of multidrug resistance protein 1 (MRP1), causing an efflux

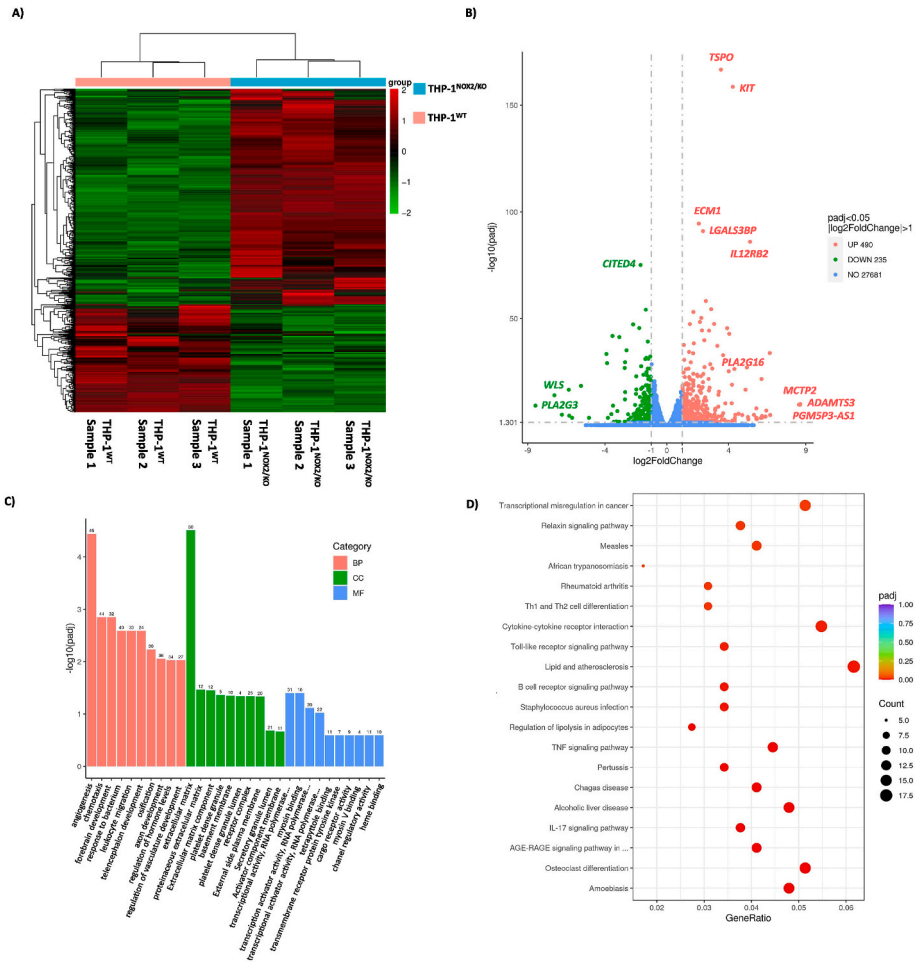

Fig. 7. NOX2 deletion triggers transcriptomic changes in THP-1 cells. A transcriptomic analysis by RNAseq was performed in NOX2-deleted THP-1 (THP-1$^{NOX2/KO}$) and wild type (THP-1^{WT}) cells. A) Heatmap representation of transcriptomic differences. Colours represent normalized gene expression by log_2(FPKM+1). B) Volcano plot representation of the differentially expressed genes ($|log_2FC|>1$ and $p_{adj} < 0.05$). 235 genes were downregulated against 490 overexpressed. C) GO terms that demonstrate significant alterations. D) Altered KEGG pathways. GeneRatio expresses the ratio of the number of differentially expressed genes in the KEGG pathway to the total number of differentially expressed genes. BP: biological processes; CC: cellular components; MF: molecular functions.

glutathione out of the cell [40]. Definitively, that would be a possibility to be tested in our system. In addition, the downregulation of glutathione synthesis in THP-1$^{NOX2/KO}$ cells, which is supported by the accumulation of glutathione precursors, could be also responsible. Leukemic cells are more sensitive to glutathione inhibition than normal cells [41]. Indeed, Craig T. Jordan's group has recently demonstrated that cysteine is essential for the survival of LSCs, which have an OXPHOS-dependent metabolism and whose activity depends on the levels of glutathionylation of complex II [42]. This would suggest the case for using NOX2 inhibitory treatment to sensitise AML cells.

In addition to the differences in the metabolome, THP-1$^{NOX2/KO}$ cells also showed profound transcriptomic differences with control cells. Analysis of these data reflected alterations of pathways related to immune response and cellular signalling, which is in line with the involvement of NOX2 in these biological processes. Regarding metabolism, several KEGG terms were related to lipid metabolism. Moreover, we found up to 12 genes involved in lipid catabolism overexpressed in THP-1$^{NOX2/KO}$ cells, what agrees with a higher dependence on lipids as source of energy. Of note, it seems that AML cells are particularly capable of reprograming lipid metabolism, which seems a new hallmark of this disease with prognosis value [43,44]. Our results show that NOX2 deletion induces a deep metabolism rewiring, reflected by seahorse, metabolome and transcriptome analyses.

Finally, we tested the clinical relevance of our experimental data. We found 30 DEGs involved in metabolism in THP-1$^{NOX2/KO}$ cells, which can segregate AML patients according to their *CYBB* expression, and more importantly that constitute a powerful prognosis tool, that predicts patient survival with great accuracy. This, added to our previous report [24], and others colleagues results [22,23,25], supports the relevance of NOX2 and metabolism for AML prognosis.

4. Conclusions

Improving AML management and treatment is hampered by the great heterogeneity of this neoplasia. A deeper understanding of the differences among AML patients could be instrumental for the development of specific AML-subtypes treatments. In this regard, metabolism and the enhanced production of ROS are emerging as promising targets. Previous reports support the relevance of NADPH oxidases in the control of metabolism. However, different roles have been described for each family member depending on the cellular context and the molecular mechanisms involved need to be refined. Here we have analysed whether NOX2 levels are important for AML metabolism control. Our Seahorse analyses show that NOX2 deletion in AML cells induces a profound change in metabolism manifested by a reduction in glycolysis and basal respiration, which is also reflected by metabolomic, transcriptomic and intracellular signalling changes. Moreover, our gene expression analyses support a strong correlation between NOX2 and metabolism involved genes, which can be harnessed for patient classification and survival prediction. In summary, our data reinforce the use

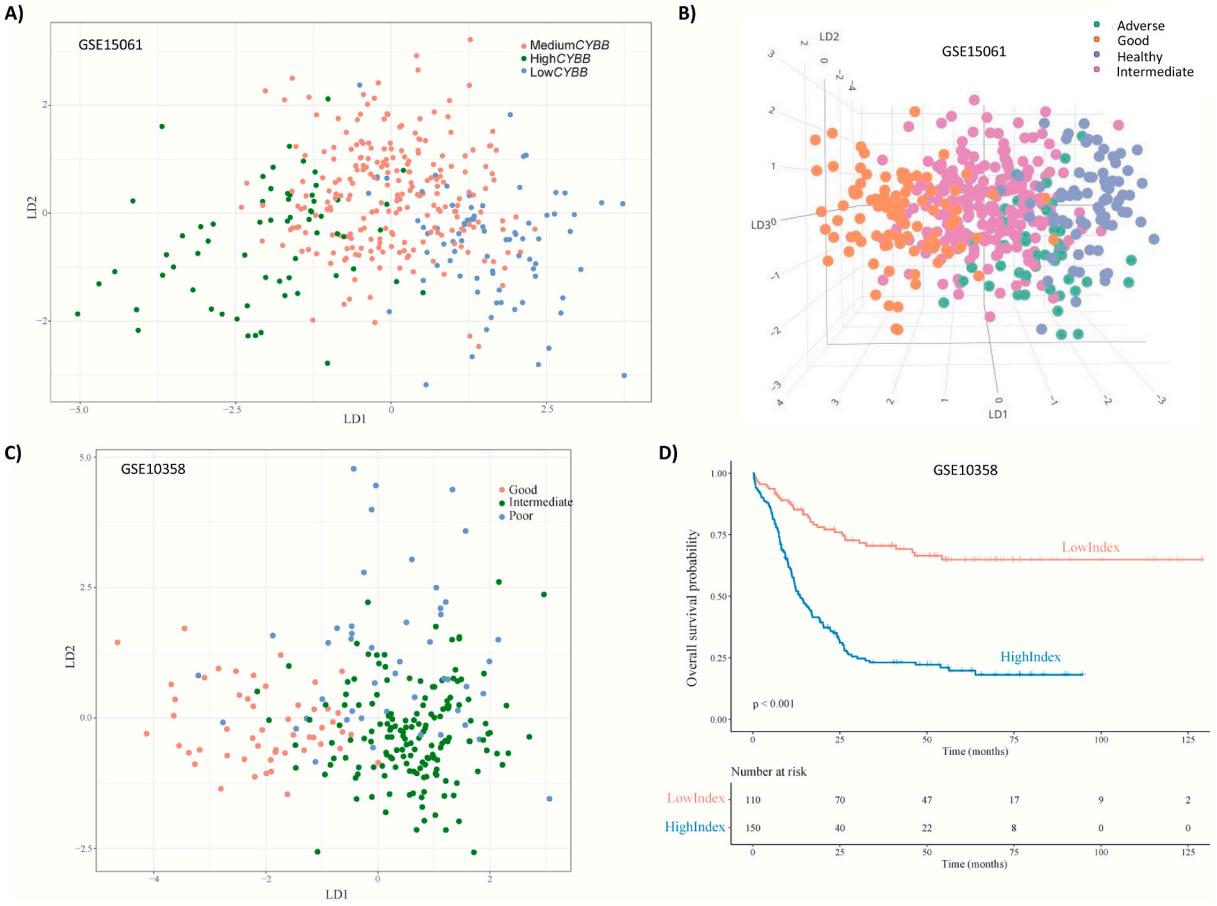

Fig. 8. 30 metabolic genes differentially expressed in NOX2-deleted THP-1 cells display AML patient classification and survival prediction ability. Considering the expression of the 30 genes listed in Supplementary Table 5, the following statistical approaches were carried out: A) LDA where *CYBB* expression groups separation is shown for GSE15061 (n = 449). B) LDA where prognosis group separation is shown GSE15061 (n = 449). C) LDA where prognosis group separation is shown for training dataset GSE10358 (n = 260). D) Kaplan-Meier overall survival (OS) curves of EI groups (High-Index and Low-Index) in 260 samples from the GSE10358 dataset.

of metabolism as a prognosis tool in AML, and more importantly strongly points to NOX2 as a crucial regulator of AML metabolism. Given the AML heterogeneity, it could be surmised that different metabolisms could be found among AML patients, and NOX2 might play prevalent role in this scenario. Future studies will delve into the underlying mechanisms, which could contribute to improve AML patients managing and treatment.

5. Materials and methods

5.1. Lentiviral silencing

Silencing was performed by lentiviral transduction and checked by RT-qPCR (Supplementary Fig. 1B) as previously [45]. Target sequences are listed in Supplementary methods.

5.2. Gene deletion by CRISPR-Cas9

Oligonucleotides targeting *CYBB* exon 4 (Supplementary methods) were annealed with a tracrRNA sequence fluorescently labelled (Alt-R® CRISPR-Cas9 tracrRNA, Fisher Scientific). THP-1 cells were nucleofected using a Neon™ Nucleofection System (1450V pulse for 10 ms) and then single-cell separation was performed by flow cytometry cell sorting. NOX2 deletion was verified by flow cytometry (Supplementary Fig. 2A), and by measuring NADPH oxidase activity (Supplementary Fig. 2B). Positive clones were characterised by genomic DNA sequencing (Supplementary Fig. 2C).

5.3. Glucose uptake

$2.0x10^5$ cells were incubated during 1h at 37 °C in glucose-free medium (RPMI glucose-free, Sigma-Aldrich, R1383) supplemented with 10 % FBS and 100 μM 2-NBDG (2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose). Glucose uptake was measured by flow cytometry.

5.4. Seahorse analysis

ATP Production (ATP Rate Assay), mitochondrial respiration (Mito Stress Assay) and substrate usage (Substrate Oxidation Assay) were measured in a Seahorse XFe24 Analyzer following the manufacturer's instructions. 100,000 cells were seeded per well pre-treated with 0.08 mg/ml Cell-Tak (Corning™). Inhibitors concentrations used were: 1.5 μM oligomycin, 0.5 μM rotenone + actinomycin, 2.5 μM BAM15, 2 μM UK5009, 4 μM Etomoxir and 3 μM BPTES. Seahorse Analytics (Agilent Technologies) was used for data analysis.

5.5. Metabolome analyses

Metabolite identification and quantitation was performed by 1H Nuclear Magnetic Resonance (NMR) (Supplementary methods).

5.6. Redox status analysis

50 million cells were incubated at a density of 1million cells/ml. Cells were harvested avoiding rough processing that could induce ROS production. Cells were centrifuged for 5 min at 1500 rpm at 4 °C and washed 3 times with 10 ml of cold PBS. The pellets were frozen at −80 °C. Subsequently, the cells were disintegrated by 5 freeze/thaw cycles in hypotonic Tris buffer. GSH content was determined by measuring the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma, Lyon, France) to 5-thio-2-nitrobenzoic acid (TNB) as previously [46]. Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase were measured as previously described [47].

5.7. Transcriptomics

mRNA library preparation was based on poly A enrichment. Paired-end 150 nucleotide length sequencing was performed on a NovaSeq 6000 from Illumina (40 million total reads). Sequencing and data analyses were carried out by Novogene using GRCH38 genome as a reference. RNAseq data is publicly available in the SRA database from NCBI with the submission localizer PRJNA977859.

5.8. AML public database analyses

GSE15061 [48] was selected as the training database, while for validation GSE14468 [49] and GSE10358 [50] were used (Supplementary methods). Linear Discriminant Analysis, Index Generation and survival analysis were conducted as previously [24].

5.9. Statistical analysis

Statistical significance (p-value <0.05) between groups was determined via Student's t-test and ANOVA (for quantitative parametric variables); Mann–Whitney U and Kruskal–Wallis tests (for quantitative nonparametric variables). A false-discovery rate correction (FDR) was used for metabolites analysis (FDR-adjusted-p < 0.05 for significance). The Kaplan–Meier method was used to construct overall survival (OS) curves, and the log-rank test was used to assess the statistical significance of the survival curves between groups (p-value <0.05).

Authorship contributions

CI performed experiments, analysed data, assemble figures and wrote the manuscript. MRG and CGC performed experiments and analyse data. JLS, CSB and JSY provided reagents and revised the manuscript. BEH and JV performed metabolomic analysis. CG performed redox status analyses. JM, PM, provided reagents, designed metabolome and redox status experiments and revised the manuscript. AHH conceived and designed experiments, analysed data and wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.10.013.

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